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RESEARCH ARTICLE

Fructose, glucose and fat interrelationships with metabolic pathway regulation and effects on the gut microbiota

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ABSTRACT

The purpose of this 30-day feeding study was to elucidate the changes, correlations, and mechanisms caused by the replacement of the starch content of the AIN-93G diet (St) with glucose (G), fructose (F) or lard (L) in body and organ weights, metabolic changes and caecal microbiota composition in rats (Wistar, SPF). The body weight gain of rats on the F diet was 12% less ($P = 0.12$) than in the St group. Rats on the L diet consumed 18.6% less feed, 31% more energy and gained 58.4% more than the animals on the St diet, indicating that, in addition to higher energy intake, better feed utilisation is a key factor in the obesogenic effect of diets of high nutrient and energy density. The G, F and L diets significantly increased the lipid content of the liver (St: 7.01 ± 1.48; G: 14.53 ± 8.77; F: 16.73 ± 8.77; L: 19.86 ± 4.92% of DM), suggesting that lipid accumulation in the liver is not a fructose-specific process. Relative to the St control, specific glucose effects were the decreasing serum glucagon (–41%) concentrations and glucagon/leptin ratio and the increasing serum leptin concentrations (+26%); specific fructose effects were the increased weights of the kidney, spleen, epididymal fat and the decreased weight of retroperitoneal fat and the lower immune response, as well as the increased insulin (+26%), glucagon (+26%) and decreased leptin (–25%) levels. This suggests a mild insulin resistance and catabolic metabolism in F rats. Specific lard effects were the decreased insulin (–9.14%) and increased glucagon (+40.44%) and leptin (+44.92%) levels. Relative to St, all diets increased the operational taxonomic units of the phylum Bacteroidetes. G and L decreased, while F increased the proportion of Firmicutes. F and L diets decreased the proportions of Actinobacteria, Proteobacteria and Verrucomicrobia. Correlation and centrality analyses were conducted to ascertain the positive and negative correlations and relative weights of the 32 parameters studied in the metabolic network. These correlations and the underlying potential mechanisms are discussed.

KEYWORDS

nutrients, metabolome, metabolism, microbiota, network, organs

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INTRODUCTION

An alarming incidence of metabolic illnesses (obesity and type 2 diabetes) has been observed over the past four decades in the United States (Mokdad et al., 2001) and also in other

affluent societies. This rise has occurred parallel to sedentary lifestyle, the consumption of foods of high energy density and the extensive use of high-fructose sweeteners. An increasing number of evidences suggest that, among other nutritional factors, high fructose consumption may play a significant role in obesity (Bray et al., 2000) and the type 2 diabetes epidemic (Ang and Yu, 2018).

High-fat diets are a strong contributor to the obesity epidemic and related co-morbidities (Mendoza et al., 2007); however, there are data suggesting that 'replacing carbohydrates with any fat, but particularly polyunsaturated fat, will lower triglyceride (TG), increase high-density lipoprotein (HDL) cholesterol, and lower blood pressure, but have no effects on fasting glucose in normal volunteers or insulin sensitivity, as assessed by euglycaemic hyperinsulinaemic clamps' (Clifton, 2019).

Another important factor influencing obesity is the composition of the intestinal microbiota. Microbiota transplantation from an overweight adult twin to germ-free mice led to increased body fat mass, while mice receiving the lean twin microbiota maintained normal weight (Ridaura et al., 2013). The microbiota of genetically obese and lean mice on the same carbohydrate-rich diet was significantly different in the reduced abundance of Bacteroidetes and increased abundance of Firmicutes in obesity (Ley et al., 2005). It is a documented fact that diets influence both the gut microbiota (Turnbaugh et al., 2009) and the immune system (Cianci et al., 2018); however, the effects of specific macronutrients need further clarification.

The aim of this study was to determine the special effects of the nutrients glucose, fructose, and lard replacing the starch content of the AIN-93G diet (control), at constant nutrient/energy ratios, on feed intake, body weight gain, feed conversion ratio, immune response, metabolomics and the distal gut microbiota and their metabolic interrelationships. While there are other studies looking at the effects of nutrients on each of the parameters cited, this is, to our knowledge, the first comprehensive study investigating the correlations and metabolic network significance among all these parameters. A more complete understanding of these interrelationships may contribute to unravelling metabolic syndrome associated disease and nutrient interactions.

MATERIALS AND METHODS

This experiment was approved by the Animal Welfare Committee of the University of Veterinary Medicine, Budapest, Hungary. Ethics approval number: 53/2013 in agreement with the permission of the Hungarian Scientific Ethics Council for Animal Research (project no. 22.1/5/003/2010).

Animals and diets

Thirty-two 10-week-old male Wistar SPF rats, weighing 200–275 g, were divided into four groups with similar average starting body weights (avg. 241.71 ± 17.66 g). The animals were individually housed in wire-bottom cages [light cycle 12 h on (7 a.m.–7 p.m.) 12 h off (7 p.m.–7 a.m.)]. The experimental diets were fed for 30 days. Water and feed were provided *ad libitum* during the experiment.

Design of diets

The AIN-93G formula of the American Institute of Nutrition (Reeves, 1997) was used as a starch-containing control diet (St diet). In two diets the starch content of this formula was replaced by either glucose or fructose (G and F diets) (Table 1). In the case of the fourth diet the starch content of

Table 1. Composition of the experimental diets*

	Starch (St)	Glucose (G) (g and %)	Fructose (F)	Lard (L)	
				(g)	(%)
Casein	10.00	10.00	10.00	10.00	15.65
Milk protein isolate	10.00	10.00	10.00	10.00	15.65
Cellulose	5.00	5.00	5.00	5.00	7.83
AIN93VX mineral premix	3.50	3.50	3.50	3.50	5.48
AIN93VX vitamin premix	1.00	1.00	1.00	1.00	1.57
Cyst, met, choline	0.50	0.50	0.50	0.50	0.78
Corn oil	5.00	5.00	5.00	5.00	7.83
Starch	65.00				
Glucose		65.00			
Fructose			65.00		
Lard				28.89	45.22
Sum total	**100.00**	**100.00**	**100.00**	**63.89**	**100.00**
Calculated crude protein content (g/100 g diet)	16.89	16.89	16.89		26.43
Calculated energy content (MJ GE/100 g diet)	1.60	1.60	1.60		2.50
Protein/Energy ratio (g/MJ)	10.55	10.55	10.55		10.55

GE = gross energy; *The energy and protein contents of the diets were calculated on the basis of the energy and the protein contents declared by the raw material manufacturer.

the AIN-93G formula (65 g/100 g diet = 1,086.8 kJ) was replaced by an energetically equivalent quantity of lard (28.89 g = 1,086.8 kJ) (L diet). Finally, the different ingredients of the L diet were expressed as a percentage of the total weights of the calculated formula (Table 1).

Replacing carbohydrate with lard significantly increased the energy density of the feed (1.6 versus 2.5 MJ GE/100 g diet), but the protein and other nutrients/energy ratios remained identical with that of the other three carbohydrate-containing diets (10.55).

This significant difference in energy density at constant nutrient/energy ratios allowed us to estimate the influence of dietary energy density on feed and energy intake and on feed conversion ratio.

Blood, plasma and serum samples

Blood, plasma or serum samples were collected in glass tubes (heparinised for plasma), kept on ice, and then centrifuged (4 °C, 10 min, 492 RCF) to obtain the plasma or serum fraction.

Tested parameters

The following parameters were investigated in this experiment: feed intake, body weight gain (BWG), eviscerated body weight (EVSCBW), feed/gain, organ weights (liver, kidney, spleen and adrenals), the epididymal and retroperitoneal white adipose tissues, serum glucose, fructosamine, total cholesterol, TG, lactate dehydrogenase (LDH), insulin, glucagon, leptin, antibody titre and the caecal microbiota.

Body weight, eviscerated body weight (EVSCBW), feed intake and organ weights

Body weight and feed consumption were measured three times a week, between 8:00 and 10:00 in the morning.

On the 30th day of the trial the rats were anaesthetised with sodium pentobarbital (35 mg/kg BW) and exsanguinated via the *vena cava caudalis* between 8 and 11 a.m. following a sequence that randomised the treatment groups. After the removal of the viscera the EVSCBW and the weights of the liver, kidneys, adrenals, epididymal and retroperitoneal fat pads were measured.

Biochemical parameters

The glucose, total cholesterol, triglyceride (TG) and fructosamine concentrations, and LDH activity of the serum samples were determined by an automated fluid chemical analyzer (Olympus 400, Olympus, Hamburg, Germany) in the Diagnostic Laboratory of the University of Veterinary Medicine, Budapest, Hungary.

Hormones

Insulin, glucagon and leptin concentrations of the blood serum were analysed by a ROCHE Cobas e 411 fully automated immunoassay analyzer, using Rat Leptin ELISA Kit (Sigma, RAB0335), Rat Ins1/Insulin ELISA Kit (Sigma, RAB0904) and Rat Glucagon EIA Kit (RayBioTech, P06883) for serum, plasma and cell culture supernatant, in the Biochemical Laboratory of DRC Ltd. (Drug Research Centre, Balatonfüred).

Immune response

On the first and 14th day of the experiment, the animals were immunised with 0.2 mL ovalbumin solution (50 μg ovalbumin + 100 μL incomplete Freund's adjuvant (IFA) + 100 μL PBS/rat) intraperitoneally. The antibody titres of the blood samples (collected on the 14th and 30th days of the experiment) were analysed using ELISA (Vucskits et al., 2010).

DNA extraction and metagenomics library preparation

Caecal contents were collected soon after exsanguination. The samples were stored at 5 °C until DNA extraction, which took place within 5 h after sampling. To study the effect of macronutrients on the microbiota, a shotgun DNA sequencing method was used.

One gram aliquots of caecal content from each of the eight rats per treatment group were pooled and analysed. Total DNA was extracted from the isolated caecal contents using the ZR Fecal DNA Kit from Zymo Research. The isolated total genomic DNA samples were sequenced using an Ion Torrent PGM platform (Life Technologies, Thermo Scientific) according to the manufacturer's recommendations. Fragment libraries were generated using Ion Xpress Plus Fragment Library Kit. Ion Shear Plus Reagents Kit was implemented for adapter ligation and nick translation. Platinum PCR SuperMix, ION Library TaqMan qPCR and Ion PGM 200 Xpress template kits were used for library amplification, quantification and for the emulsion PCR, respectively. Sequencing was performed on Ion 318 chips, generating 150 k – 520 k high-quality reads per sample with an average read length of 208 ± 87 bp.

Bioinformatic analysis

Quality-based filtering and trimming was performed by the Trimmomatic sequence analysis tool (Bolger et al., 2014); (with settings HEADCROP:12 CROP:320 LEADING:3 TRAILING:3 SLIDINGWINDOW:30:15 MINLEN:50) only readings longer than 50 bp were retained. The *Rattus norvegicus* genome sequences as host contaminants were filtered out by Bowtie2 (version 2.3.5) (Langmead and Salzberg, 2012) with very-sensitive-local setting minimising the false positive match level (Czajkowski et al., 2019) in further metagenome classification. The remaining reads after deduplication by VSEARCH (version 2.15.2) (Rognes et al., 2016) were taxonomically classified using Kraken2 (version 2.0.9-beta) (k = 35) (Wood et al., 2019) with the NCBI non-redundant nucleotide database (Pruitt et al., 2005). The Kraken reports were converted to biom structure by the tool kraken-biom. For further steps of the taxon classification data processing the phyloseq and microbiota R packages were used. The alpha diversity was expressed by the Shannon index.

Statistical analysis

For the interpretation of the results, as a gold standard, the data from rats on the St diet were used as a basis for comparison.

Statistical analysis was performed using SPSS version 18.0 (IBM SPSS Statistics, 2016), which included ANOVA followed by *post hoc* LSD and polynomial regression analysis. In the presented networks the nodes are the variables. The networks were built based on the correlation of the parameters. All parameter pairs were correlated, and the statistic was tested (Myles and Wolfe, 1973). Below the $P < 0.05$ threshold, the variables are connected independently by the correlation direction. These connections are represented by edges in the graphs. The analysis was performed by the R-environment (R Core Team, 2016) (R: A Language and Environment for Statistical Computing). Centrality (the weight of the nodes) was analysed by the yEd Graph Editor (yEd Graph Editor, 2012). In network science, centrality is a measure of the importance of the nodes composing the network.

Because we measured pooled samples for DNA extraction (8 rats/treatment group and 1 g of caecal content from each rat), the data of microbiome investigations are not supported by statistical analysis. The heatmap was generated by R-package pheatmap (Kolde, 2018).

RESULTS AND DISCUSSION

Feed and energy intake, body weight gain, feed conversion ratio and abdominal white adipose tissues

Feed and energy intake. The data indicate (Table 2 and Fig. 1) that the feed and energy intakes of rats on the three

Table 2. Specific effects of nutrients and energy density on the studied parameters

	Starch (St)	Glucose (G)	Fructose (F)	Lard (L)
		Nutritional parameters ± STD		
Energy density of the diets (kJ/g gross energy)	16.32	16.32	16.32	25.52
Feed intake (g/100 g BW/day)	7.80 ± 1.10^{a}	7.53 ± 0.23^{a}	7.68 ± 0.89^{a}	6.35 ± 0.37^{b}
Energy intake (GE kJ/100 g BW/day)	124.3 ± 17.6^{a}	122.6 ± 3.8^{a}	125.5 ± 14.6^{a}	162.8 ± 9.6^{b}
BW gain (g/100 g BW/day)	1.33 ± 0.25^{a}	1.30 ± 0.17^{a}	1.17 ± 0.36^{a}	2.11 ± 0.49^{b}
Feed/gain (g/g)	5.86 ± 0.36^{a}	5.78 ± 0.76^{a}	6.56 ± 0.79^{a}	3.01 ± 0.60^{b}
Eviscerated body weight (EVSCBW) (g)	278.1 ± 9.9^{a}	264.5 ± 19.2^{b}	259.7 ± 20.2^{b}	320.0 ± 15.3^{c}
		Fat pads		
Epididymal (% of EVSCBW)	0.82 ± 0.31^{a}	0.84 ± 0.28^{a}	0.91 ± 0.26^{a*}	1.32 ± 0.41^{b}
Retroperitoneal (% of EVSCBW)	0.82 ± 0.38^{a}	0.76 ± 0.40^{a}	0.59 ± 0.27^{a*}	1.48 ± 0.56^{b}
		Organ weights		
Liver (g/100 g BW)	3.47 ± 0.27^{a}	3.95 ± 0.48^{b}	4.30 ± 0.65^{b}	3.50 ± 0.31^{a}
Kidney (g/100 g BW)	0.68 ± 0.04^{a}	0.68 ± 0.06^{a}	0.77 ± 0.06^{b}	0.63 ± 0.04^{c}
Spleen (g/100 g BW)	0.27 ± 0.05^{a}	0.30 ± 0.05^{ab}	0.34 ± 0.07^{b}	0.28 ± 0.06^{a}
Adrenals (mg/100 g BW)	22.00 ± 6.00^{a}	24.88 ± 8.94^{b}	25.00 ± 5.29^{b}	21.75 ± 2.6^{a}
		Chemical composition of the liver		
Ash (% of DM)	6.67 ± 0.84^{a}	7.24 ± 2.24^{a}	7.13 ± 1.25^{a}	5.90 ± 1.28^{b}
Ether Extract (% of DM)	7.01 ± 1.48^{a}	14.53 ± 8.77^{b}	16.73 ± 8.77^{b}	19.86 ± 4.92^{b}
Crude protein (% of DM)	74.52 ± 3.32^{a}	68.56 ± 7.97^{b}	67.02 ± 6.18^{b}	69.12 ± 4.49^{b}
N-free extract (% of DM)	11.63 ± 3.80^{a}	8.73 ± 7.49^{ab}	9.63 ± 4.57^{a}	4.70 ± 3.20^{b}
		Metabolomics		
Serum glucose (mmol/L)	7.58 ± 1.23^{a}	9.82 ± 3.11^{b}	8.36 ± 1.51^{c}	8.45 ± 1.27^{bc}
Fructosamine (µmol/L)	455.4 ± 43.6^{a}	488.3 ± 88.0^{a}	398.4 ± 38.2^{b}	464.4 ± 37.6^{a}
Total cholesterol (mmol/L)	1.59 ± 0.18^{a}	1.27 ± 0.27^{b}	1.61 ± 0.28^{a}	2.10 ± 0.32^{c}
Triglyceride (mmol/L)	1.11 ± 0.29^{a}	1.44 ± 0.54^{b}	1.91 ± 0.56^{c}	0.93 ± 0.20^{d}
LDH (U/mL)	1.75 ± 0.60^{a}	1.64 ± 0.84^{ab}	1.84 ± 0.78^{ab}	1.53 ± 0.37^{b}
Insulin (µg/L)	1.86 ± 0.56^{a}	1.97 ± 0.74^{ab}	2.74 ± 1.85^{ab}	1.69 ± 0.46^{b}
Glucagon (pg/mL)	4.08 ± 3.20^{a}	2.37 ± 1.28^{b}	5.14 ± 4.20^{c}	5.73 ± 2.03^{c}
Leptin (ng/mL)	1.87 ± 0.78^{abc}	2.34 ± 1.25^{a}	1.40 ± 0.36^{b}	2.71 ± 1.33^{c}
		Immune response		
Antibody titre (14th day) (Log2)	10.64 ± 1.41	11.42 ± 1.18	10.52 ± 0.99	9.59 ± 1.66
Antibody titre (30th day) (Log2)	14.02 ± 1.69^{ab}	14.36 ± 1.98^{b}	13.27 ± 1.06^{ac}	12.89 ± 1.04^{c}
		Ratios		
Insulin/Glucagon	0.46	0.83	0.53	0.30
Insulin/Leptin	0.99	0.84	1.96	0.62
Glucagon/Leptin	2.18	1.01	3.67	2.11

STD = standard deviation. Different letters indicate statistically significant differences ($P < 0.05$) between the treatment groups; *indicates significant difference between the epididymal and the retroperitoneal fat pads in rats on fructose diets.

carbohydrate diets (St, G, and F) were statistically identical, suggesting that the satiety effects of these carbohydrate diets were the limiting factor of feed intake. In contrast, the rats on the energy- and nutrient-dense L diet consumed significantly less feed (–18.7%) and more energy (+30.9%) than the rats on the starch control diet (Table 2).

This result is consistent with the data of Stubbs et al. (2000), who suggest that energy density exerts profound effects in constraining energy intake as a major factor and influences appetite. Dietary macronutrients exert differential effects on food and energy intake and the energy densities of diets are more effective at increasing energy intake than at decreasing food intake. In the current experiment the percentage increase of energy density of the lard diet over the starch diet was numerically identical with the improvement of body weight gain (58.4%).

Body weight gain. Feeding the F diet resulted in 12% less body weight gain than that of rats on the St diet, and the difference was close to the statistically significant level ($P = 0.120$) (Fig. 1). This effect is in accordance with the result of Mahmood et al. (2019), i.e. that fructose in the drinking water decreases the body weight gain of rats compared to water-treated control animals.

In contrast to this, rats on the nutrient- and energy-dense L diet gained 58.4% more than the control animals on the starch diet. This result is consistent with that reported by Bhandari et al. (2011).

Feed conversion ratio. The feed conversion (feed/gain) ratio (Table 2 and Fig. 1) for the three carbohydrate diets was not different statistically. The feed conversion ratio of the lard diet was significantly better than that of the carbohydrate diets.

The facts that the rats on lard diet consumed 16% less feed, 31% more energy and gained 58.4% more than the control animals on the starch diet indicate that better nutrient utilisation may be a key factor in explaining the obesogenic effect of diets of high energy density.

Eviscerated body weight. Compared to the St diet, the G and F diets significantly decreased (–4.9 and –6.6%, respectively), while the L diet increased (+15.1%) the EVSCBW, suggesting that in rats on the G and F diets the distribution of the synthesised body mass shifted toward the visceral fat stores (Fig. 2).

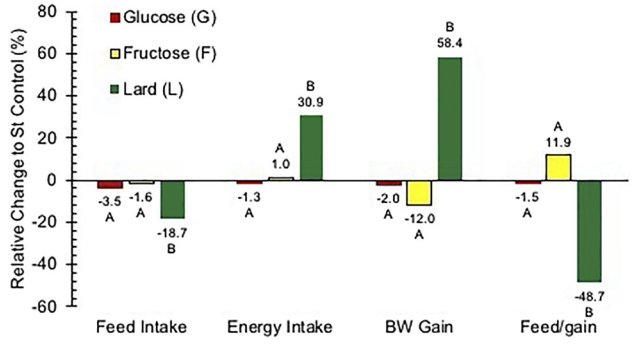

Fig. 1. Effect of diets on feed intake, energy intake, body weight gain and feed/gain ratio. Different letters indicate significant differences among the treatment groups at $P < 0.05$

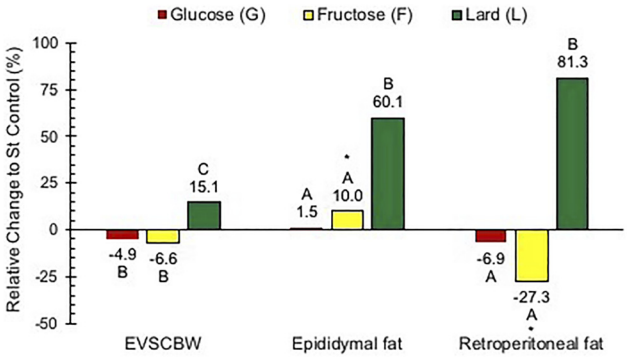

Fig. 2. Effect of diets on eviscerated body weight (EVSCBW) and epididymal and retroperitoneal fat pads. Different letters indicate significant differences among the treatment groups at $P < 0.05$. *indicates significant difference between the weight change of the epididymal and retroperitoneal fat pads

Retroperitoneal and epididymal adipose tissues. Table 2 and Fig. 2 summarise the specific effects of macronutrients on the abdominal adipose tissues.

The combined weight of epididymal and retroperitoneal fat pads of rats on the isocaloric glucose or fructose diets showed no difference from that of rats on the starch diet. This is consistent with the fact that there was no significant difference in weight gain either among these treatment groups. Diet G did not influence, while diet F slightly but not significantly increased the weight of the epididymal (+10%) fat pad. Both monosaccharides decreased the weight of retroperitoneal adipose tissues (glucose: –6.9%), and the effect of fructose was close to the significant level (–27.3%; $P = 0.098$). Other studies reported just the opposite of these results, namely that fructose significantly increased the weight of the retroperitoneal adipose tissue over the control values (Zubiria et al., 2016). They observed that the retroperitoneal adipose tissue increased after eight weeks on a fructose-enriched diet; this depended on the combined accelerated adipogenesis and adipocyte hypertrophy, partly due to the direct effect of fructose on adipocyte precursor cells.

In an 8-week-long experiment with rats on isocaloric high-fructose or control diets, Crescenso et al. (2014) detected a significantly increased weight of the epididymal and mesenteric white adipose tissues of rats in the high-fructose group.

In the current experimental model, the weight of epididymal and retroperitoneal adipose tissue was influenced by the F diet in the opposing direction, and the difference between them was significant ($P < 0.001$).

Compared to the carbohydrate diets, the L diet of high energy density induced a significant increase in the weights of both the epididymal (+60.1%) and the retroperitoneal (+81.3%) white adipose tissue. Other authors (Talarico

et al., 2020) reported that a diet high in unsaturated fat increased the weight of the retroperitoneal adipose tissue (12.9 ± 3 g) over the control (6.7 ± 1 g) and the saturated fat diet groups (7.7 ± 1 g). Both high-fat groups increased the weight of the epididymal adipose tissue (control diet: 5.8 ±1 g; saturated fat diet: 8.2 ± 1 g; unsaturated fat diet: 10.9 ± 3 g).

In their review, Bjørndal et al. (2011) suggest that 'adipose tissue metabolism is closely linked to insulin resistance, and differential fat distributions are associated with disorders like hypertension, diabetes, and cardiovascular disease'.

Specific effects of macronutrients on organ weights

Our working hypothesis was that fructose would affect organs that have GLUT-5 fructose transporter and the ketohexokinase (KHK-C) enzyme; these organs are the small intestine, the liver, the kidney and the pancreas (Diggle et al., 2009). The other organs and the muscles have the KHK-A enzyme which has a much higher Km than KHK-C, and consequently the cells of these organs can metabolise fructose when its plasma concentration is high, making these latter organs less sensitive to the detrimental effects of fructose.

The G diet significantly increased the weight of the liver and the adrenals but not that of the spleen (Table 2). The F diet significantly increased the weight of the liver, kidney, spleen, and adrenals; however, the latter two organs have no GLUT5 transporter, thereby partially negating our working hypothesis.

The function of the spleen is to filter the blood, detect potentially dangerous microorganisms and thereby elicit an immune response (Mebius and Kraal, 2005). A high dietary level of monosaccharides (glucose or fructose) may influence the gut microbiota and may increase intestinal permeability, lipopolysaccharide (LPS) absorption and the development of metabolic endotoxaemia (Do et al., 2018), thereby resulting in spleen enlargement. Increased plasma uric acid levels, inherent in fructose metabolism, may also cause adrenal enlargement. The hypothalamic–pituitary–adrenal axis can be stimulated by the purine metabolites that are rapidly formed during the metabolism of fructose. Indeed, there is evidence that a high-fructose diet significantly increases serum uric acid (Caliceti et al., 2017) and corticosterone (Harell et al., 2015) levels and may alter the function of the hypothalamic–pituitary–adrenal axis.

Earlier findings further strengthen this speculation, considering that adenosine and its metabolites (except for hypoxanthine) significantly increase plasma corticosterone level (Szabó et al., 1995) and may play a significant role in the control mechanisms of the hypothalamic–pituitary–adrenal axis (Szabó and Bruckner, 1995). Based on clinical and animal studies, excess glucocorticoids have been implicated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Patients with NAFLD seem to have a subtle chronic activation of the hypothalamic–pituitary–adrenal axis leading to a state of subclinical hypercortisolism (Papanastasiou et al., 2017).

Currently there is no explanation as to why the glucose diet increased adrenal weight. Fructose and sucrose may also induce renal hypertrophy and tubulointerstitial disease in rats (Kretowicz, 2011).

The lard diet of high energy density increased the eviscerated body weight of rats (Table 2) but did not influence the weight of the liver, spleen, or adrenals; interestingly, it significantly decreased the weight of the kidney. This contradicts the result of Altunkaynak et al. (2008) who found significantly increased kidney volume and histopathological renal deformities in rats on a high-fat diet; the fat source of their diet was unclear. It is clear that the effects of a lard diet on the weight of the studied organs are strikingly different from those of glucose and fructose.

Chemical composition of the liver

To get more insight into the specific effects of macronutrients on the liver, the chemical composition of the liver was analysed (Table 2 and Fig. 3). The effects of the G and F diets were practically the same.

The most significant effects of the G and F diets were the dramatic increases of the liver fat content and the significant decrease of liver protein content.

The decrease of nitrogen-free extract, most likely glucose and glycogen (Szabó et al., 2018) and the change of ash concentrations were significant only in rats on the L diet. In rats fed the G, F or L diet, significantly more lipid accumulated in the liver than in the starch-fed control rats. The order of magnitude of liver fat content was starch < glucose, < fructose and < lard (Table 2 and Fig. 3). In rats fed the G and F diets, lipid accumulation in the liver was likely due to the increased level of *de novo* lipogenesis.

Fallon and Kemp (1968) obtained a similar result when investigating hepatic lipid synthesis in liver homogenates from rats fed a high-carbohydrate diet and a diet high in corn oil. They concluded that 'high-carbohydrate diets fed to rats for 6 days increased the triglyceride synthesis'. Diets high in starch were less effective than diets containing high levels of glucose, sucrose, or fructose. Corn oil did not alter the TG synthesis of hepatocytes. It is well documented that dietary fructose, sucrose and high-fructose corn syrup tend

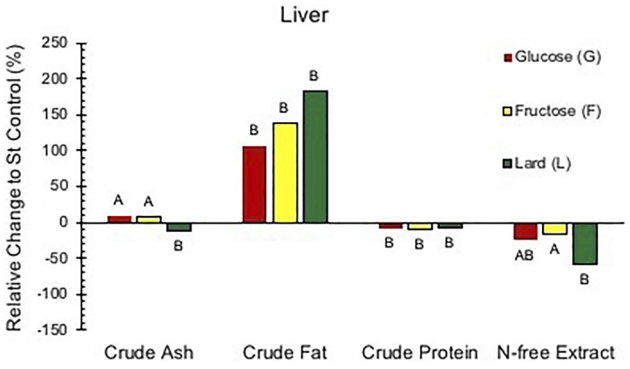

Fig. 3. Effect of diets on the chemical composition of the liver. Different letters indicate significant differences among the treatment groups at $P < 0.05$

to induce fatty liver; however, it was suggested that the development of fatty liver takes a longer time period (8–24 weeks) (Jensen et al., 2018).

An interesting question that may be asked is why the fat content of the liver was significantly higher in rats on the G diet than in the starch-fed control animals; the carbohydrate content of the diets was similar, and the absorbed carbohydrate was glucose in both cases.

A simple answer might be that in a given unit of time much more glucose may enter the liver than from the slower absorbable starch, causing higher intracellular glucose and triose concentrations as well as increased lipogenesis.

However, Katz and McGarry (1984) suggest that lipid synthesis in rodents may be due to the fact that 'the glucose administration acts mainly as a trigger, but the hexose serves only indirectly (via lactate or glycogen) as a precursor for this process in the liver'. Pilkis and Granner (1992) suggest that the uptake of glucose by hepatocytes is mediated through the glucose transporter GLUT2, which does not require insulin for activation. The consequence of this could be that glucose enters the liver cells without insulin regulation and thereby may result in very high intracellular glucose concentration.

Glucose is first converted to glycogen and once glycogen stores are replenished, glucose enters the glycolysis pathway and thereby provides carbons for the tricarboxylic acid (TCA) cycles and *de novo* lipogenesis (Weickert and Pfeiffer, 2006). High glucose and insulin administration have also been shown to inhibit fatty acid oxidation (Randle, 1998).

It was also interesting to note that the N-free extract (carbohydrate) content of the liver of rats on G, F or L diets was lower (–24.9, –17.2 and –59.6%, respectively) than in the group fed the St diet; however, the difference was statistically significant only in rats on the L diet.

This is consistent with the data of Rémésy et al. (1978), who conditioned rats to 42 or 79% carbohydrate containing diets and noted that the portal vein glucose concentration was 8.8 mM in the rats eating the 42% carbohydrate diet but net hepatic glucose output was still positive. Animals consuming the high-carbohydrate diet exhibited hepatic glucose uptake, which was at the expense of a much higher portal vein glucose concentration (13.6 mM).

According to Merino et al. (2020), fructose is transported by GLUT5 through the brush border membrane. In the cytosol of enterocytes, it is rapidly phosphorylated by KHK-C, leading to a rapid depletion of intracellular ATP level. A pool of phosphorylated fructose is partially or totally metabolised. The non-phosphorylated fructose is released across the basolateral membrane into the portal circulation by apical GLUT2. Blakemore et al. (1995) reported that the GLUT5 transporter is also expressed in the basolateral membrane of the human intestine, suggesting that non-phosphorylated fructose can easily pass through the enterocytes and reach the liver, where it can be metabolised.

Hepatic fructose uptake is not regulated and there is no negative feedback. Because KHK-C phosphorylates fructose to fructose-1-P, it bypasses the rate-limiting step of glycolysis and yields precursors that can be used for gluconeogenesis and *de novo* lipogenesis.

According to the excellent paper of Jang et al. (2018), the fate of orally administered fructose depends on the quantity of consumed fructose. Low doses of fructose (<0.5 g/kg BW) were ~90% cleared by the intestine, with only trace amounts of fructose found in the portal blood; however, elevated concentrations of glucose, lactate, and glycerate derived from fructose were noted. In the portal vein, they observed that most dietary fructose has already been converted into glucose and various organic acids (lactate, glycerate, TCA intermediates, and amino acids) which can be good substrates for lipogenesis. Therefore, the effect of low doses of fructose on the liver lipogenesis may be similar to that of glucose.

According to Zhao et al. (2020), dietary fructose is converted to acetate by the gut microbiota, and this supplies lipogenic acetyl-CoA independently of ATP citrate lyase. They suggest 'a two-pronged mechanism that regulates hepatic lipogenesis, in which fructolysis within hepatocytes provides a signal to promote the expression of lipogenic genes, and the generation of microbial acetate feeds lipogenic pools of acetyl CoA'.

This speculation is in line with that reported here and the findings of Johnston et al. (2013) who reported that there was no difference between the effects of high-fructose and high-glucose diets on liver triacylglycerol or biochemistry in healthy overweight men. High doses of fructose (≥1 g/kg BW) overwhelm intestinal fructose absorption and clearance, resulting in fructose reaching both the liver and the colonic microbiota.

Fructose also rapidly decreases the intracellular adenylate energy charge (AEC) (ATP + 0.5 ADP + ATP + ADP + AMP), resulting in increased uric acid production due to the activation of AMP deaminase (Khitan and Kim, 2013). Son et al. (2020) reported that serum uric acid levels were positively associated with the dyslipidaemia components of serum, e.g. total cholesterol, TG and LDL cholesterol (LDL-C) levels, whereas serum HDL-C levels were inversely related. The fructose-induced depletion of liver adenine nucleotides inhibited protein synthesis in the liver (Mäenpää et al., 1968), suggesting that apoprotein synthesis may also be inhibited and the ineffective packing of TG into nascent very low density lipoprotein (VLDL) may be a factor in the pathomechanism of fructose-induced liver steatosis.

Reviewing isotopic tracer studies, Sun and Empie (2012) concluded that although fructose is a potent lipogenic substrate, the observed fat synthesis from fructose carbons is quantitatively minor compared with other pathways of fructose disposal. Only 0.05% and 0.15% of fructose was converted to *de novo* fatty acids and TG-glycerol at 4 h, respectively. The origin of the hepatic lipids in rats fed the fructose diet remains to be determined.

As Table 2 and Fig. 3 show, the L diet induced almost three times more lipid in the liver than the St diet. The liver derives fatty acids primarily from three sources: uptake of free fatty acids (FFAs) from the blood, chylomicron remnant uptake, and *de novo* lipogenesis.

In this study, the most important factor of lipid accumulation in the liver was noted in the high-fat L diet (lard

45.2% + corn oil 7.8%) without carbohydrate. Because the absorbed triglycerides bypass the liver, entering the systemic circulation directly by the lymphatic circulation, the high serum and liver TG concentration probably originates from chylomicron remnants and esterified and non-esterified fatty acids.

High-fat diets strongly inhibit hepatic lipogenesis and contribute to the accumulation of TG in the liver (Ferramosca, 2014). Liu et al. (2015) reported that a high-fat diet induced markedly higher total FFA serum concentration compared to the control diet in the fed state. In an earlier experiment, Bruckner et al. (1998) demonstrated that a diet low in n-3 essential fatty acids and a poor-quality protein in the diets of cats were required to induce hepatic lipidosis, suggesting that both protein quality and fatty acid composition of the diet may play a significant role in the pathomechanism of fatty liver syndrome. In rates fed a fructose diet, citrulline supplementation prevented hypertriglyceridaemia and attenuated liver fat accumulation (Jegathesan et al., 2015).

Specific effects of macronutrients on serum metabolites and hormones

Serum glucose. Table 2 and Fig. 4 show the effect of macronutrients on serum metabolites. The G, F and L diets significantly increased the postprandial serum glucose level over the value of rats on a St diet (glucose: +29.6%, fructose: +10.3% and lard: +11.6%).

According to Wang et al. (2010), the average postprandial blood glucose concentration of normal Wistar rats is in the range between 5.65 mmol/L and 7.90 mmol/L. Assuming a 40% haematocrit, this value corresponds to 9.4 ± 2.72 and 13.2 mmol/L serum glucose concentrations (Amara et al., 2018). Therefore, the postprandial serum glucose concentrations in the current study were in the normal range (St: 7.58 ± 1.23, G: 9.82 ± 3.11, F: 8.36 ± 1.51 and L: 8.45 ± 1.27 mmol/L serum).

Serum fructosamine. Fructosamine determines the glycated fraction of total serum protein and reflects one- to two-week changes in blood glucose. Figure 4 shows that neither the G nor the L diet influenced the fructosamine level significantly. However, the F diet decreased it significantly (–12.5%), suggesting that a 4-week feeding of these nutrients was not long enough to damage, or interfere with, carbohydrate metabolism. Another explanation may be that when balanced diets are fed, as in the current experiment, the negative effect of F or L on carbohydrate metabolism is minimal or does not occur.

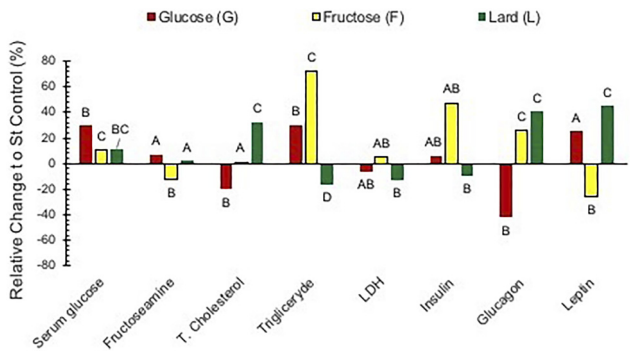

Fig. 4. Specific effects of the St, G, F and L diets on serum metabolites and hormones. Different letters indicate significant differences among the treatment groups at $P < 0.05$

Serum cholesterol. G significantly ($P < 0.05$) decreased (–19.9%) and L significantly ($P < 0.05$) increased (+32.3%) the total cholesterol level of the serum, but F did not change it relative to the St control group (Fig. 4). Other researchers have also reported similar results for fructose (Mamikutty et al., 2014; Köseler et al., 2018).

Serum triglyceride. In current study, both the G and the F diets increased, while the L diet decreased the serum TG level significantly ($P < 0.05$) (Fig. 4). This is substantiated by the findings of others (Nishina et al., 1993) in that mice normally have low plasma TG levels which are further lowered by high-fat diets. According to Byers and Friedman (1960), the liver is the major source of plasma TG. Schaefer et al. (2009) suggested that dietary glucose and fructose differentially affected lipid and glucose homeostasis; they mention that in short-term controlled feeding studies, fructose significantly increased postprandial TG levels and had little effect on serum glucose concentrations, whereas dietary glucose had the opposite effects.

Serum LDH. The serum LDH activity was determined in this study in order to see if the diets have a hepatotoxic effect (Table 2). Figure 4 shows the relative changes of LDH activity compared to the St control group. G slightly decreased, while F slightly increased serum LDH activity, but neither of these changes were significant. The difference between the G and the F groups is close to the significant level ($P = 0.11$). This suggests that the F diet induced no cell damage during the four-week feeding period. The L diet significantly decreased the LDH activity, suggesting that during this experimental time period lard had no toxic effect on the liver.

Serum insulin. The data of Table 2 and Fig. 4 show that relative to the St diet, neither the G nor the F diet had significant effects on the serum insulin concentration; however, in the latter group serum insulin was 47.7% higher than the control value and close to the significant level ($P = 0.105$). The L diet significantly (–8.8%; $P < 0.05$) decreased serum insulin. This result is consistent with the data reported in a previous experiment with cold-stressed rats (Szabó et al., 2018), notably that safflower oil reduced plasma insulin concentration compared to both fasted rats and rats fed a balanced diet. Zavaroni et al. (1980) reported similar findings in rats feed fructose for 7 days, which resulted in an increase in the insulin response to an oral carbohydrate challenge, as well as to a loss of normal insulin sensitivity.

Serum glucagon. As shown in Table 2 and Fig. 4, the G diet decreased while the F and the L diets significantly increased the serum glucagon concentration. In the rats fed diet F the serum glucose and insulin concentrations were also increased, suggesting early signs of insulin resistance. This is supported by the opinion of Elliott et al. (2002) that fructose causes insulin resistance syndrome. Another mechanism that may be involved is that alpha cells have GLUT5 transporters (Sato et al., 1996) and KHK enzyme (Giroix et al., 2006), the fructose entering the alpha cells is rapidly phosphorylated and thus may acutely deplete ATP and inorganic phosphate (P_i) resulting in a cytosolic energy deficit, which may in turn trigger glucagon release. The L diet also increased glucagon concentration.

In contrast to this, in a previous experiment with cold-stressed rats, safflower oil significantly decreased the plasma glucagon concentration. The exact effects of lipids on plasma glucagon concentration are unclear since both inhibitory and stimulatory effects have been reported by others (Edwards et al., 1969; Gilon et al., 2014). Most likely the environment and the dietary fatty acid composition of the fat fed may explain some of these opposing findings.

Serum leptin. In this experiment the G and L diets increased, while the F diet decreased the postprandial leptin concentration of the serum (Table 2 and Fig. 4). As opposed to the current study, Jian-Mei et al. (2008) reported that fructose consumption significantly increased the serum leptin and uric acid levels in rats and induced a significant increase of leptin secretion by upregulating the expression of the obese (*ob*) gene in adipose tissue. Quercetin and rutin (plant flavonoids, antioxidants), as well as allopurinol reduced hyperleptinaemia and inhibited leptin secretion from adipose tissue. Like in the current study, they also found increased serum insulin concentration. Others (Shapiro et al., 2008) reported that chronic fructose consumption caused leptin resistance, while serum leptin levels, weight, and adiposity were the same as in the leptin-responsive control rats.

Insulin/glucagon (I/G), insulin/leptin (I/L) and glucagon/leptin (G/L) ratios. Three hormonal ratios were calculated from the serum hormone concentrations to better understand their interrelationships. Figure 5 clearly shows that G and F have different effects on I/G, I/L and G/L ratios, which indicates that although fructose and glucose carry the same quantity of gross energy, they have different hormonal effects. Glucose elicits an anabolic hormonal response, while fructose has an indirect catabolic effect by the small I/G and high I/L and G/L ratios. G and L diets also have different effects on the I/G and G/L ratios. However, in the case of the L diet, the synthetic pathways may also be inhibited by the low insulin and high glucagon levels.

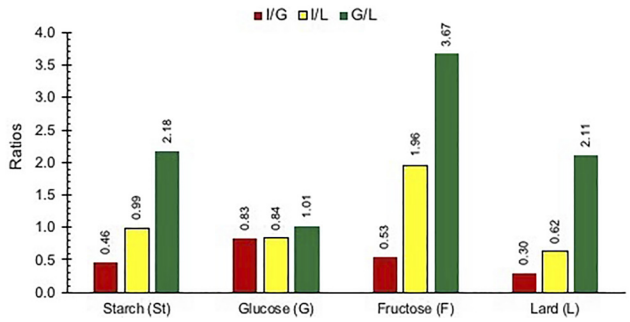

Fig. 5. Effect of diets on insulin per glucagon (I/G), insulin per leptin (I/L) and glucagon per leptin (G/L) ratios

Specific effects of the St, G, F, and L diets on the immune response and the microbiota

Immune response. To better understand the interrelationship between the nutrients and the immune response of an organism, its physiology, metabolism and the changes of the intestinal microbiota have to be considered. In this experiment, the nutrients did not have a significant effect on the humoral immune response by the 14th day after the first immunisation (Table 2 and Fig. 6).

However, after the second immunisation, by the 30th day of the experiment, the G diet increased, while the F and L diets significantly decreased the antibody titre. In an earlier experiment, Szabó et al. (2018) demonstrated that glucagon correlated significantly with the serum glucocorticoid level (not determined in this experiment). The immunosuppressive effect of increased glucagon (Kevorkov et al., 1987) and corticosterone (Coutinho and Chapman, 2011) concentration may explain the lower serum antibody titre in the rats fed the F diet. This is also supported by other authors who reported that the feeding of high-fructose (Prince et al., 2017) or high-fat (Namvar et al., 2016) diets was associated with elevated levels of corticosterone.

Akkermansia and *Escherichia* were correlated positively (r = 0.964 and 0.960, respectively) and *Fusobacterium* negatively (r = –0.972) to the serum antibody titre, suggesting that there is a balance between the stimulating and inhibiting effects of the microflora on the immune function (Fig. 6).

The association between the gut microbiota and immunity has been shown to influence the development of major

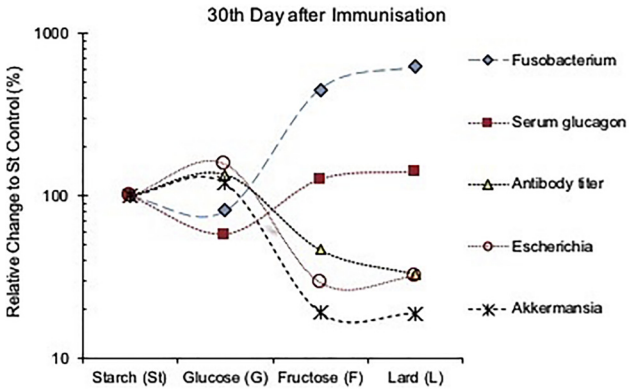

Fig. 6. Effect of diets on serum glucagon, antibody titre and the genera *Fusobacterium, Escherichia*, and *Akkermansia* (dotted lines do not indicate a continuum)

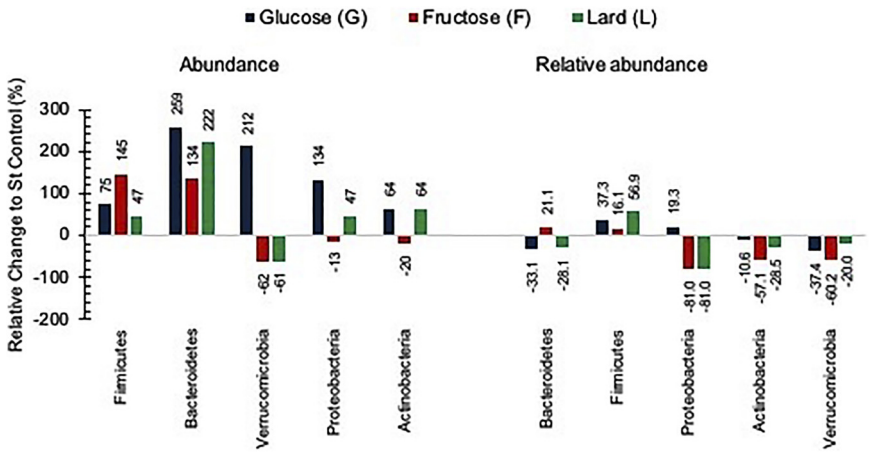

Fig. 7. Effect of the St, G, F and L diets on the caecal microbiota. Depending on how the data are expressed (abundance or relative abundance), different results may be obtained

components of the host's innate and adaptive immune system and may contribute to the pathogenesis of immune-mediated disorders (Zheng et al., 2020).

It was reported that certain fusobacteria can inhibit human T-cell responses (Nsho et al., 2016). For example, *Fusobacterium nucleatum* may mediate the initiation and progression of colorectal cancer by inhibiting host anti-tumour immunity (Borroni et al., 2019).

It is clear from these data that nutrients can significantly influence the host organism's actual concentration of hormones, its metabolism, the intestinal microbiota, and the immune response. Further studies should be carried out to determine these interrelationships and to administer appropriate nutrients in accordance with the therapeutic goals.

Effect of the St, G, F, and L diets on the microbiota. The effects of the St, G, F, and L diets on the taxonomic diversity of the microbiota, at different levels of taxonomy, is presented in Table 3. A very conspicuous difference is noted in the total number of reads per g of caecal content for glucose, fructose, or lard diets (39,056, 30,141 and 30,610, respectively), where the reads were twice as high as in the starch-fed group (14,924). At the different taxonomic levels (genus, family, order, class, phylum) the diversity was generally lower in rats fed the fructose diet compared to the glucose- and the lard-fed groups (Table 3).

Table 3 also indicates that there are significant differences in the number of phyla detected in the caecal contents of the rats fed the St, G, F and L diets (26, 35, 35 and 28, respectively). The Shannon diversity index was 4.526, 4.069, 3.347 and 4.275 for the St, G, F and L diets, respectively.

In the rats fed the G diet, the operational taxonomic units (OTUs) of the five most abundant phyla were increased, while F feeding increased only Firmicutes and Bacteroidetes and decreased the other three phyla (Fig. 7). The effect of L on Firmicutes and Bacteroidetes was similar to that seen in rats fed the G diet; however, the OTUs of Verrucomicrobia decreased similarly as in the F group. Furthermore, the L diet increased the number of OTUs in Proteobacteria (+47%) and Actinobacteria (+64%). Figure 7 shows that depending on the manner in which the data are expressed (abundance or relative abundance), different results may be obtained.

If the data are expressed as relative abundance (Table 4 and Fig. 7), the sum of the reads represents 100% in each treatment group.

The phyla Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria and Actinobacteria represented 97.54, 98.48, 98.47 and 97.55% of the entire genome for St, G, F and L diets, respectively.

Firmicutes and Bacteroidetes were the two predominant phyla, contributing 39.9% and 37.83% in the St group, 26.69% and 51.94% in the G group, 48.32% and 43.92% in the F group, and 28.67% and 59.35% in the L group, respectively. Verrucomicrobia, Proteobacteria and Actinobacteria were the next three most dominant phyla, accounting for 9.21%, 8.31% and 2.29% in the St group, 10.98%, 7.43% and 1.43% in the G group, 1.75%, 3.57% and 0.91% in the F group, and 1.75%, 5.94% and 1.83% in the L group, respectively.

Figure 7 indicates that, as compared to the St diet, the G and the L diets decreased, while the F diet increased the proportion of Firmicutes. In contrast, the G, F and L diets increased the relative proportion of Bacteroidetes in the entire genome. The Verrucomicrobia/Firmicutes ratio was increased slightly by the G diet and decreased dramatically by the F and L diets. The direction of the dietary effects on the phyla Proteobacteria and Actinobacteria was negative and more or less identical.

Another possibility for evaluating the effect of diets on the microbiota is to calculate the ratios of the different phyla to Firmicutes (Fig. 8). According to this interpretation, the effects of starch and glucose are very similar and the trend, when comparing fructose and lard diets, is also similar; however, the magnitudes of the ratio changes were different.

The lowest ratios to Firmicutes were those of Verrucomicrobia and Proteobacteria in the F and L groups. The

Table 3. Effect of the starch, glucose, fructose, and lard diets on the diversity of operational taxonomic units (OTUs)/gram caecal content

	Number of OTUs/gram caecal content			
	Starch	Glucose	Fructose	Lard
Shannon diversity index*	4.526	4.069	3.347	4.275
Total reads	14,924	39,056	30,141	30,610
Genus	732	883	676	851
Family	295	329	286	302
Order	145	152	138	141
Class	73	69	64	68
Phylum	26	35	35	38
	Number of OTUs/g caecal content at the phylum level			
Firmicutes	5,953	10,425	14,564	8,777
Bacteroidetes	5,647	20,285	13,238	18,168
Verrucomicrobia	1,374	4,288	528	536
Proteobacteria	1,241	2,903	1,076	1,819
Actinobacteria	342	560	275	561
	Ratios to Firmicutes			
Firmicutes/Bacteroidetes	1.05	0.51	1.10	0.48
Firmicutes/Verrucomicrobia	4.33	2.43	27.58	16.38
Firmicutes/Proteobacteria	4.80	3.59	13.54	4.83
Firmicutes/Actinobacteria	17.41	18.62	52.96	15.65

*The Shannon diversity index is commonly used to characterise species diversity in a community. The value depends on the number of species – the more species, the higher the diversity.

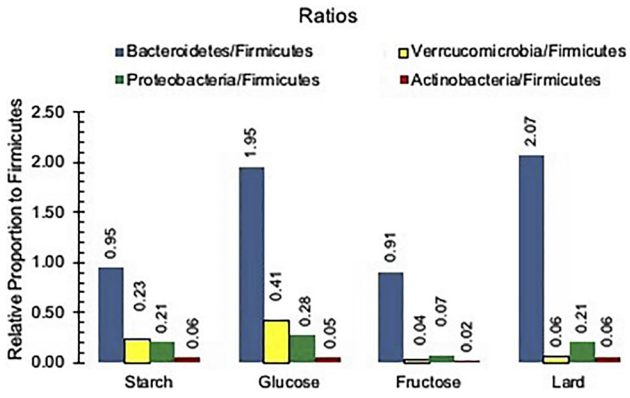

Fig. 8. Phylum ratios based on the relative abundance of operational taxonomic units (OTUs)

Bacteroidetes to Firmicutes ratios were similar in the St and F or the G and L groups.

Figure 9 depicts the effects of the experimental diets on the relative proportions of different genera in the caecal microbiota. Compared to St, G and L decreased, while F increased the proportion of *Lactobacillus*.

The numbers of *Bacteroides, Alistipes, Butyricimonas* and *Phascolarctobacterium* were increased by the G and L diets. F slightly increased the proportion of *Bacteroides* but did not markedly influence *Alistipes* and *Butyricimonas*; however, F decreased the proportions of Parabacteroides and *Phascolarctobacterium* in the caecal microbiota. G increased but F and L decreased the proportion of *Akkermansia* and *Escherichia* in the caecal microbiota.

The G, F, and L diets decreased the proportions of *Ruminococcus, Eubacterium, Streptococcus, Clostridioides, Clostridium* and *Muribaculum*. G and F decreased the proportions of *Lachnoclostridium* and *Oscillibacter*, while L did not influence these genera. F decreased, but G and L did not influence the proportions of *Blautia*. The effects of diets on the microbiota are numerous and interpretation of the effect of these changes on physiologic, biochemical, and clinical outcomes will require further studies.

A constructed heatmap diagram of two-way hierarchical clustering analysis consisted of the four treatment groups on the genus level of microbiota (Fig. 10). According to this, the microbiota of rats fed G and L diets was more closely related, while St and F diets were hierarchically farther away. When comparing the different carbohydrate diets, the G and F diets affect the microbiota quite differently than the St diet fed in the control group. Glucose and fructose appear to attenuate many of the microbiota genera compared to starch. It is evident from the variability of the data, as presented by the different calculations, that different outcomes might be concluded.

Connections among the caecal microbiota and the studied clinical parameters. To begin understanding which clinical indicators might be related to the microbiota changes, network analysis and centrality (weight of connected nodes) were used to ascertain the possible correlations between the microbiota and the clinical indicators studied.

Correlations among the microbiota, feed and energy intake, body weights and abdominal white adipose tissues. The feed intake, energy intake, BW gain, EVSCBW, feed/gain, epididymal and retroperitoneal fat pads and the microbes significantly correlating with these parameters are indicated in Fig. 11.

Centrality analysis showed that the retroperitoneal fat pad and the EVSCBW are the two most important nodes in

Table 4. Relative abundance of operational taxonomic units (OTUs)

Phylum	Starch (St)	Glucose (G)	Fructose (F)	Lard (L)
	Criteria of reduction			
	At least 1 genus OTU in each treatment group			
Firmicutes	39.889	26.692	48.320	28.674
Bacteroidetes	37.838	51.938	43.920	59.353
Verrucomicrobia	9.207	10.979	1.752	1.751
Proteobacteria	8.315	7.433	3.570	5.943
Actinobacteria	2.292	1.434	0.912	1.833
Per cent of entire genome	**97.54**	**98.48**	**98.47**	**97.55**
	More than 0.1% relative phylum abundance in the starch control group			
Cyanobacteria	0.402	0.269	0.149	0.268
Tenericutes	0.369	0.207	0.129	0.121
Spirochaetes	0.348	0.172	0.103	0.258
Euryarchaeota	0.281	0.179	0.076	0.199
Fusobacteria	0.275	0.195	0.697	0.947
Chloroflexi	0.181	0.064	0.027	0.085
Deinococcus-Thermus	0.101	0.049	0.060	0.049
Per cent of entire genome	**99.50**	**99.61**	**99.71**	**99.48**
	At least 1 phylum OTU in each treatment group			
Chlorobi	0.074	0.061	0.043	0.085
Aquificae	0.074	0.031	0.007	0.036
Synergistetes	0.060	0.041	0.043	0.069
Acidobacteria	0.047	0.033	0.013	0.033
Thermotogae	0.047	0.038	0.036	0.029
Planctomycetes	0.040	0.044	0.043	0.039
Chlamydiae	0.027	0.010	0.020	0.036
Deferribacteres	0.020	0.013	0.010	0.023
Chrysiogenetes	0.020	0.003	0.003	0.007
Thaumarchaeota	0.020	0.010	0.003	0.003
Crenarchaeota	0.013	0.008	0.010	0.029
Nitrospirae	0.013	0.010	0.003	0.010
Balneolaeota	0.013	0.010	0.010	0.003
Ignavibacteriae	0.007	0.020	0.003	0.029
Gemmatimonadetes	0.007	0.003	0.003	0.020
Fibrobacteres	0.007	0.005	0.003	0.010
Per cent of entire genome	**99.99**	**99.95**	**99.97**	**99.94**
	The rest of the entire genome			
Calditrichaeota	0.007	0.013	0.003	–
Dictyoglomi	0.007	0.008	–	–
Elusimicrobia	–	0.005	0.003	0.010
Kiritimatiellaeota	–	–	0.003	0.010
Armatimonadetes	–	0.003	0.010	0.007
Thermodesulfobacteria	–	0.008	0.003	0.007
Candidatus Cloacimonetes	–	–	0.003	0.007
Candidatus Gracilibacteria	–	0.003	0.000	0.007
Coprothermobacterota	–	0.005	0.003	0.003
Negarnaviricota	–	0.005	–	0.003
Caldiserica	–	–	–	0.003
Candidatus Korarchaeota	–	–	–	0.003
Per cent of entire genome	**100.00**	**100.00**	**100.00**	**100.00**

this network, influencing the network both positively and negatively. It is well known that adipose tissues play significant roles in controlling metabolism, e.g. they are important regulators of energy and glucose metabolism and act as endocrine organs secreting bioactive substances and hormones (Lou and Liu, 2016).

The negative correlations of the epididymal and retroperitoneal adipose tissues with EVSCBW are also anticipated because there is a known crosstalk between skeletal muscle and adipose tissue metabolism (Argilés et al., 2005), and visceral obesity is known to be associated with the loss of skeletal muscle mass (Kim et al., 2014). *Alistipes, Parabacteroides* and *Morganella* also occupy a relatively high position in this network. They show positive relationships with energy intake, BW gain and retroperitoneal fat pads, and negative relations with feed intake and EVSCBW. These

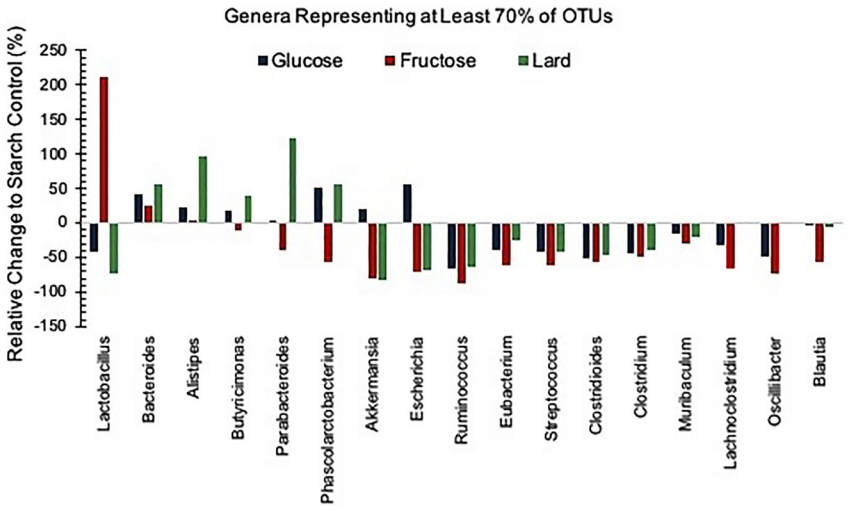

Fig. 9. Specific effect of the G, F and L diets on the caecal microbiota in genera representing 99% of the caecal microbiota

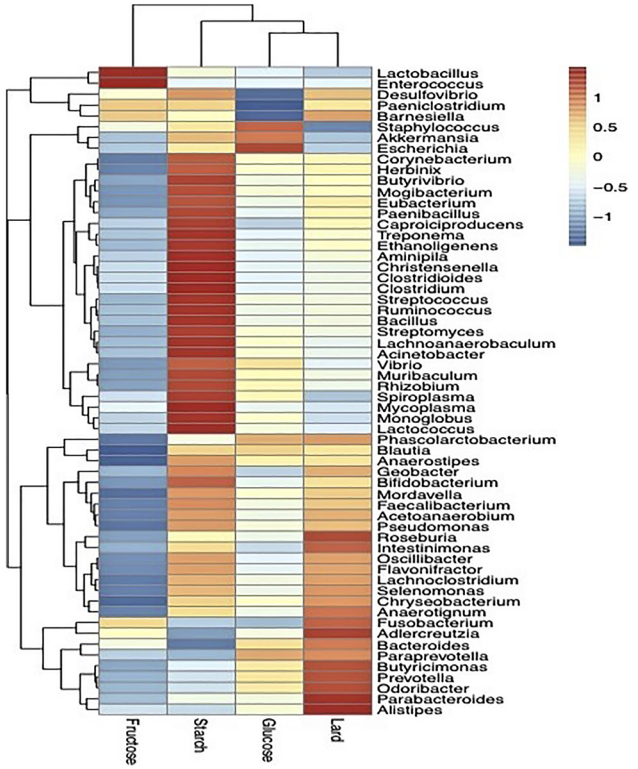

Fig. 10. Heatmap diagram of two-way hierarchical clustering analysis consisting of four treatment groups and 60 genera of the caecal microbiota of rats. The red colour represents an expression level above mean, while the blue colour represents an expression lower than the mean. On the vertical left side, the hierarchical cluster of the genera is indicated. Bottom labelling shows the treatment groups

results are similar to those reported by Zeng et al. (2019). They studied the microbiota of obese and healthy patients and found six gut microbiota markers of obesity, namely the alteration of *Bacteroides, Parabacteroides, Blautia, Alistipes, Romboustia* and *Roseburia.* In our rat model, *Adlercreutzia, Alistipes, Parabacteroides, Petrimonas* and *Morganella* showed positive or negative correlations with the obesity-related parameters.

Correlations and centrality of the microbiota and organ weights. In the current experiment, the correlation and centrality analysis revealed several possible correlations between the organ weights and the microbiota. Analysis at the genus level revealed two clubs of nodes with positive or negative connections with the microbiota (Fig. 12).

The members of the first small club are the kidney weights and three genera that show positive (*Lactobacillus*) and negative (*Proteiniphilum* and *Butyricimonas*) correlations with the weight of the kidney. Several pathological or beneficial effects of the microbiota on the liver (Brenner et al., 2015), kidney (Ramezani and Raj, 2014), cardiovascular (Tang et al., 2017) and metabolic diseases (Pascale et al., 2018) have been reported. We hypothesised a bidirectional relationship with positive and negative effects on each other.

This hypothesis is strengthened by the clinical outcomes that alterations in the microbiota may also affect kidney disease in both a positive and a negative direction and, furthermore, the abnormally functioning kidney may disrupt a balanced microbiota (Al Khodor and Shatat, 2017).

The second large club of networks indicates the relationship among the weights of spleen, liver and adrenals with the members of the caecal microbiota at the genus level. According to centrality analysis the spleen, the liver and the adrenals are in the centre of this second club of the network, showing primarily negative relations with members of the microbiota.

Correlations among the microbiota and metabolomics at genus level. In this network (Fig. 13) four smaller and one large club of nodes can be distinguished. The first is the insulin/glucagon ratio with three microbes (*Pseudomonas, Orella* and *Morganella*).

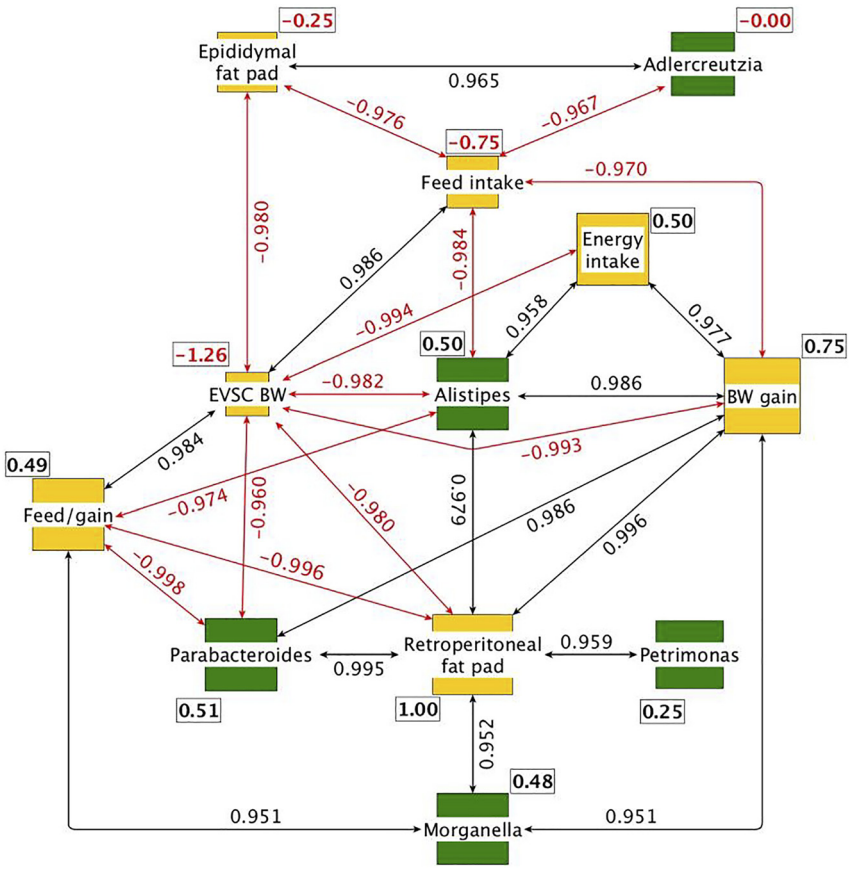

Fig. 11. Correlations among the feed and energy intake, eviscerated body weight (EVSCBW), abdominal fat pads and caecal microbiota at the genus level. Centrality indices (framed numbers) quantify the importance of a node in a given network

The second club includes serum glucagon, antibody titre, *Escherichia, Akkermansia* and *Fusobacterium*. Gram-negative microbes containing LPS in their outer membrane are known to play a significant role in the immune response of the host organism, promote pro-inflammatory cytokines, nitric oxide and eicosanoids (Abbas et al., 2012). Antibody titre showed a negative correlation with serum glucagon and *Fusobacterium* but a positive correlation with *Escherichia* and *Akkermansia*. It is well known that the antibody titre is influenced by commensal and pathogenic intestinal microbes (Belkaid and Hand, 2014).

It is believed that *Akkermansia muciniphila* protects against obesity and augments antitumour responses (Ansaldo et al., 2019). In contrast, *Fusobacterium nucleatum* suppresses anti-tumour immunity (Gur et al., 2019). More research is needed to unravel the significance of these interrelationships.

The third club includes the glucagon/leptin ratio, *Pasteurella* and *Tenerella*. There is a strong positive correlation between Pasteurella and serum fructosamine and since fructosamine is positively correlated with glucagon/leptin, these correlations may be associated with increased risk for cardiovascular disease and energy expenditure.

The fourth club contains serum glucose with negative correlations with *Desulfovibrio* and *Paeniclostridium*.

The fifth club includes serum triglyceride, serum insulin, insulin/leptin, serum leptin and serum LDH with the depicted microbiota. While all of the genera listed in Fig. 13 have negative correlations with serum triglyceride, there are both positive and negative correlations with serum insulin and the microbiota, e.g. *Faecalbaculum, Enterococcus* and *Lactobacillus* are positively correlated, while *Selenomonas* and *Proteiniphilum* are negatively correlated. While these correlations do not indicate cause and effect, they are useful in possibly interpreting how dietary changes in the microbiota might influence, or can be influenced by, clinical markers and related clinical conditions.

Correlations among the microbiota and metabolomics at the phylum level. Figure 14 shows the correlations and centrality of all studied parameters and the microbiota at the phylum level. It is interesting to note that while Firmicutes and Bacteroidetes are the predominant phyla associated with the caecal microbiota, they did not correlate significantly with each other (r = –0.729), nor with the feed and energy intake, BW gain, abdominal white adipose tissues, and serum metabolites.

The only exception was that the phylum Bacteroidetes was negatively correlated with liver N-free extracts (r = –0.962). This latter relationship suggests that nutrients increasing the glucose and glycogen content of the liver may also be in a negative correlation with Bacteroidetes. It is also known that the microbiota of obese individuals possesses a lower proportion of Bacteroidetes than that of the lean control (Clarke et al., 2012).

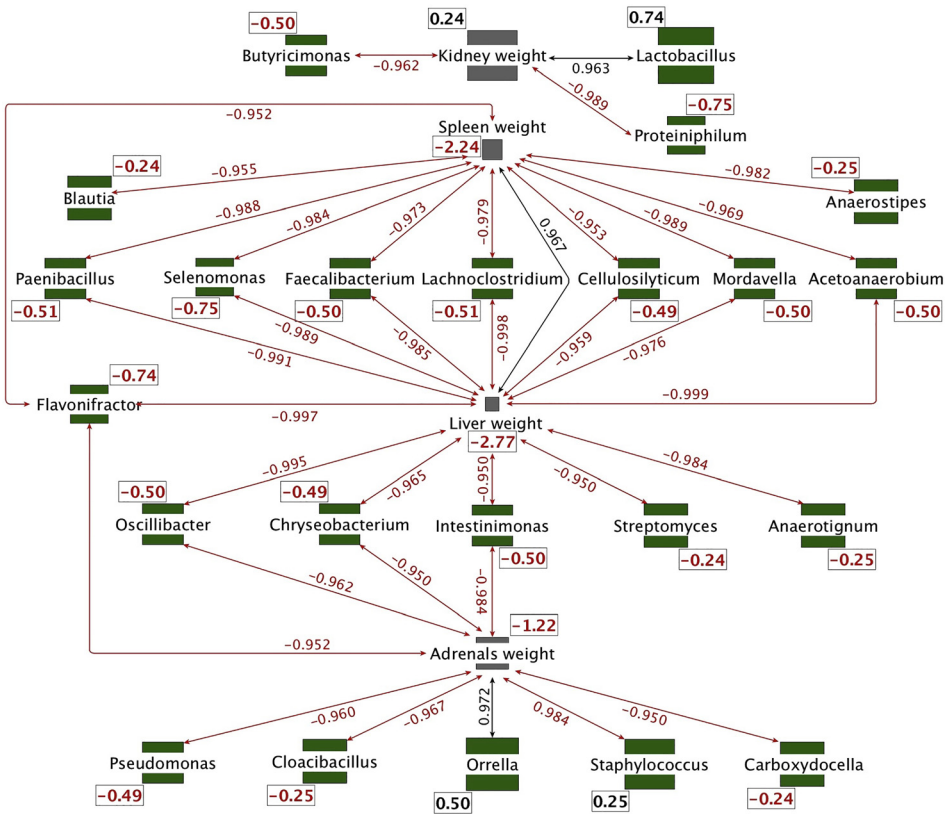

Fig. 12. Significant correlations and centrality indices (weight of connected nodes) among organ weights and caecal microbiota at the genus level. Centrality indices (framed numbers) quantify the importance of a node in a given network

It is also clear from the correlation analysis at the phylum or genus levels that the lower taxonomic levels (i.e., genus) yield more information about the specific crosstalk between the host organism, diet, and the microbiota under both healthy and pathological conditions.

Summary of the specific effects of the G, F or L diets

Table 5 summarises the direction of the significant changes induced by the experimental diets. Three out of the 13 parameters showing a significant change in the rats fed the G diet, namely the decrease of serum glucagon, leptin and glucagon/leptin ratio, were glucose-specific effects.

Relative to the data of rats on the St diet, the F diet changed significantly 17 clinical parameters and only six of them (the increased spleen and kidney weights, insulin/leptin and glucagon/leptin ratios, the decreased serum fructosamine and leptin concentrations) were effects specific to the F diet.

Among the 22 significantly changed parameters of rats fed the L diet, fifteen (the increased energy intake, BW gain and EVSCBW, the weights of the epididymal and retroperitoneal fat pads, the serum cholesterol level, the decreased feed intake, feed/gain ratio, weight of the kidney, ash and N-free extract content of the liver, insulin/leptin ratio, serum TG, insulin, and insulin/glucagon ratio) were changes specific to the L diet.

An interesting observation was that in rats fed the F diet, the weight of the epididymal (increased) and retroperitoneal (decreased) fat depot changed in the opposite directions, and the difference between them was statistically significant.

Speculations why fructose may be toxic

Based on the cited literature and our experimental data summarised in Table 5, the following general speculations and conclusions may be drawn:

1. We assume that the effects of fructose depend on the form in which it is presented, e.g., injected or per os, administered in liquid or solid form, disaccharide vs. monosaccharide, alone or with other nutrients.

The reason for the different affects of fructose administered in soft drinks compared to dry diets may be due to the higher dose administered in a given unit of time, the rapidity of absorption from the small intestine and the high concentration of fructose reaching the hepatocytes in a given unit of time.

2. The effect of a fructose diet may also depend on the gastric transit time and the ratio of other macronutrients in the diet. Fat, for example, slows down the gastric transit time, resulting in less fructose to be absorbed from the small intestine in a unit of time and lower fructose concentration in the liver. In the different

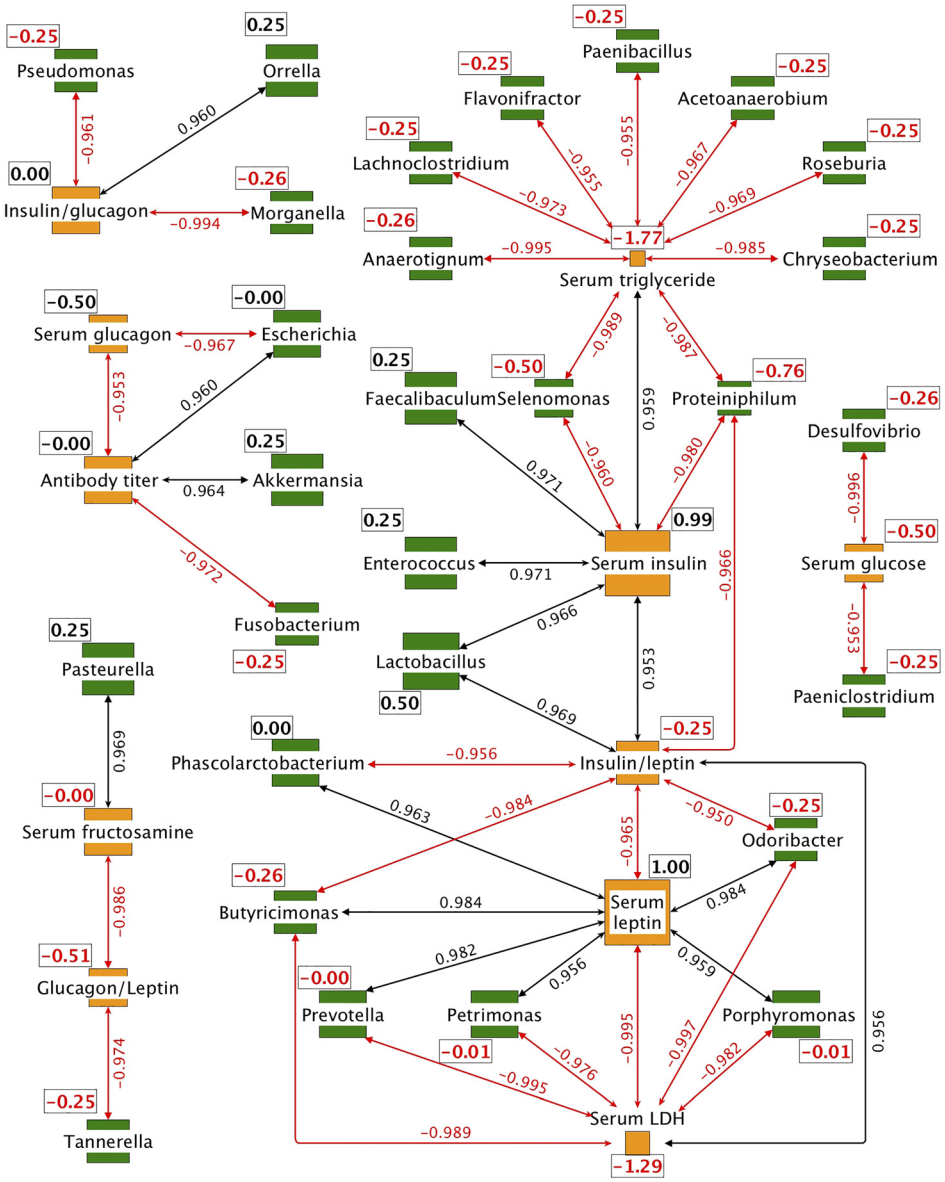

Fig. 13. Significant correlations and centrality indices (weight of connected nodes) of serum parameters and caecal microbiota at the genus level. Centrality indices (framed numbers) quantify the importance of a node in a given network

tissues having GLUT5 transporters and KHKC enzymes (e.g., enterocytes, hepatocytes, kidney cells), fructose is rapidly phosphorylated and bypasses the phosphofructokinase regulatory step in glycolysis. Only the rate, the rapidity, the dose, and the duration of the fructose load determines whether the administered fructose can pass through the shields of enterocytes and hepatocytes, and therefore this determines whether or not it will be toxic. Fructose, upon reaching the systemic circulation and the peripheral cells, may influence their metabolism depending on whether they have a GLUT5 transporter and on the type of KHK enzymes.

3. The consequences of a high intake of fructose include an immediate increase of hepatic glucose, pyruvate, and lactate production via the activation of pyruvate dehydrogenase, the esterification of nonesterified fatty acids, the increase of adenine nucleotide degradation and thereby accelerated uric acid formation with subsequent hyperuricaemia (Mayes, 1993). Hyperuricaemia may contribute to pancreatic beta cell dysfunction and death and may be implicated in the development of type 2 diabetes mellitus (Johnstone, 2015; Caliceti et al., 2017).
4. The increased triose pool induces glycogen and lipid synthesis as long as the decrease of cytosolic adenylate energy charge (AEC) = (ATP + 0.5 ADP + ATP + ADP + AMP) makes this possible. The physiological values of energy charge are somewhere between AEC = 0.7 and AEC = 0.95, stabilising at a value of 0.9 (De la Fuente et al., 2014). In a severely energy-depleted condition, the AEC drops, resulting in cell death. Chapman et al. (1972) suggested that 'growth can occur only at energy charge values above 0.8, and that viability is

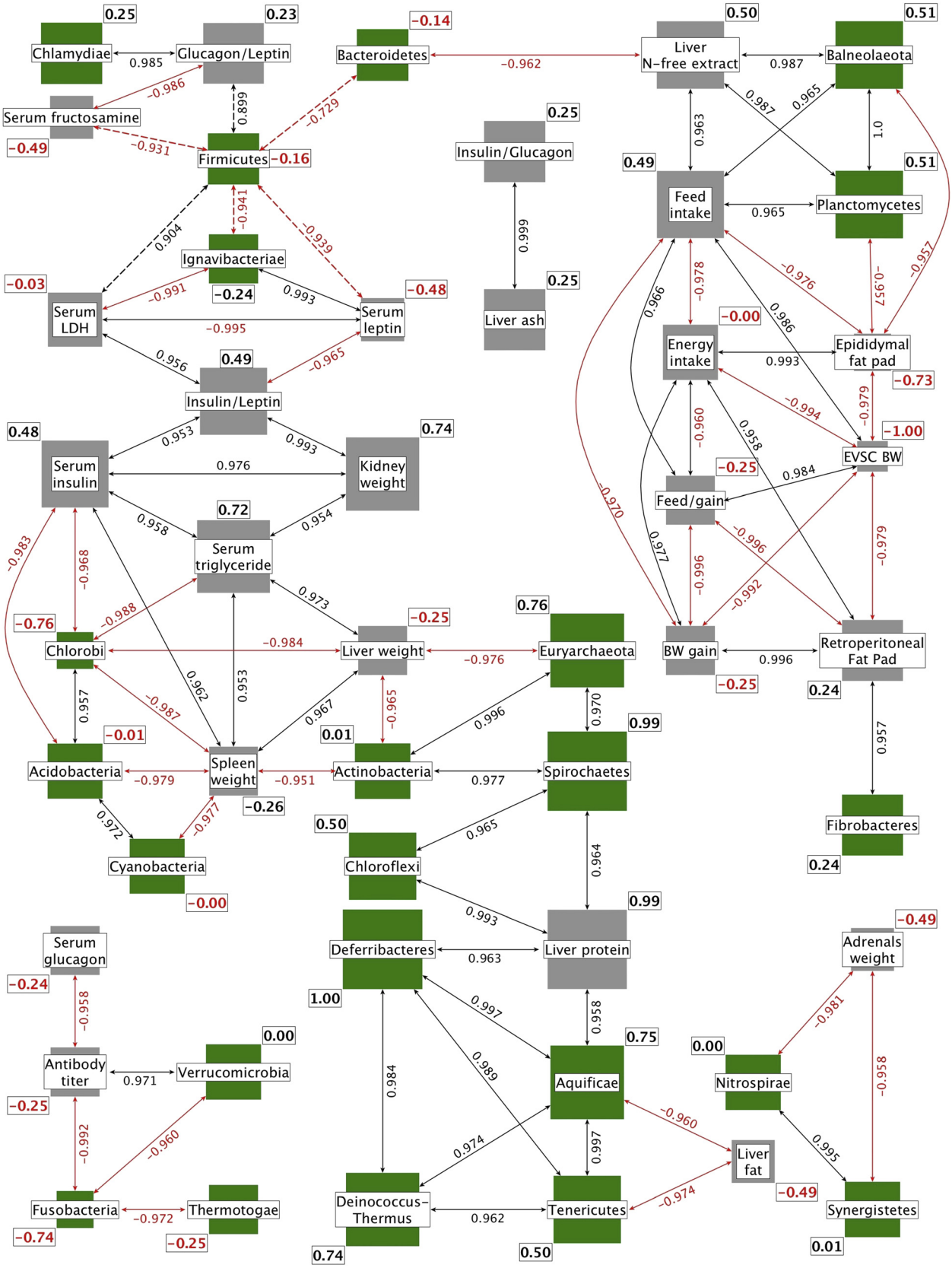

Fig. 14. Significant correlations and centrality indices (weight of connected nodes) among caecal microbiota and the measured parameters at the phylum level. Dashed line indicates non-significant correlations. Centrality indices (framed numbers) quantify the importance of a node in a given network

Table 5. Specific effects of nutrients on the studied parameters

	Starch (St)	Glucose (G)	Fructose (F)	Lard (L)
Parameters that are influenced only by the L diet				
Feed intake	–	–	–	↓
Energy intake	–	–	–	↑
Body weight gain	–	–	–	↑
Feed/gain ratio	–	–	–	↓↓
Liver crude ash	–	–	–	↓
Liver N-free extract	–	–	–	↓
Lactate dehydrogenase (LDH)	–	–	-	↓
Epididymal fat pad	–	–	↑ns*	↑↑
Retroperitoneal fat pad	–	–	↓ns*	↑↑
Parameters that *are* influenced by the F diets				
Spleen weight	–	–	↑	–
Fructosamine	–	–	↓	–
Parameters influenced by the F and L diets				
Kidney weight	–	–	↑	↓
Insulin/leptin ratio	–	–	↑	↓
Antibody titre	–	–	↓	↓
Parameters influenced by the G and F diets				
Liver weight	–	↑	↑	–
Adrenals weight	–	↑	↑	–
Glucagon/leptin ratio	–	↓↓	↑	–
Parameters influenced by G, F and L diets				
Liver crude protein	–	↓	↓	↓
Liver crude fat	–	↑	↑	↑
Serum glucose	–	↑↑	↑	↑
Serum triglyceride	–	↑	↑↑	↓↓
Insulin	–	↑	↑↑	↓
Insulin/glucagon ratio	–	↑↑	↑	↓
EVSCBW	–	↓	↓	↑
Glucagon	–	↓	↑	↑↑
Leptin	–	↑	↓	↑
Parameter influenced by the G and L diets				
Serum total cholesterol	–	↓	–	↑↑
Effect of nutrients on the caecal microbiota (phyla)				
Bacteroidetes	–	↑↑	↑	↑↑
Firmicutes	–	↓	↑	↓
Actinobacteria	–	↓	↓	↓
Proteobacteria	–	–	↓	↓
Verrucomicrobia	–	–	↓↓	↓↓

EVSCBW = eviscerated body weight. Different letters indicate statistically significant differences ($P < 0.05$) between the treatment groups; * indicates significant difference between the epididymal and the retroperitoneal fat pads in rats on fructose diets. The specific effect of a diet is marked with different colours

maintained at values between 0.5 and 0.8, and that cell death occurs at values below 0.5'. This, together with the lipid accumulation, may be the first step leading to fatty liver and subsequent cirrhosis.

5. Itoh (1983) orally administered fructose to rats. The AEC of control rats was 0.74 ± 0.02 and after the administration of fructose the AEC decreased to 0.5 within 2 min. In case of glucose administration, a similar effect was observed, however the magnitude of change was much less. In case of low energy charge the energy-dependent cytosolic synthetic pathways can be inhibited. This is consistent with the findings of Chong et al. (2007), in that, under acute postprandial conditions, little activity from radiolabelled fructose showed up in the fatty acid portion of triacylglycerols (TAG), that is, 'fatty acids are primarily from endogenous fat'; also, the incorporation of DL-leucine-1-(14)C into liver protein is almost completely inhibited (Mäenpää et al., 1968). The inhibition of lipid oxidation and of apoprotein synthesis, necessary for the lipid export, may result in liver lipid accumulation. Excess fructose consumption also affects extra hepatic adipocytes by favouring increased glutamate and fatty acid synthesis and release (Varma et al., 2015). Fructose, that is not absorbed, is a good substrate for volatile fatty acid (VFA) synthesis by the gut microbiota and the absorbed acetate is also a good substrate for hepatic lipid synthesis (Zhao et al., 2020).
6. On this basis, the following fructose paradox hypothesis may be presented: despite a high cytosolic triose concentration, ADP is not resynthesised to ATP. The resynthesis may not occur because of the following:
   a. The phosphorylation of fructose by the fructokinase reaction is rapid, whereas the aldolase B reaction is relatively slow (Champe and Harvey, 1987); the lowered availability of Pi limits the rate of ATP resynthesis. Nishi et al. (1989) have shown that perfusion of the liver with a high fructose concentration (>2 mM) causes the accumulation of fructose-1-P and the depletion of Pi.
   b. Fructose impairs the mitochondrial respiration in hepatocytes by decreasing the activities of aconitase and GOT by 35% and 47%, respectively (Madlala et al., 2018).
   c. The depletion of ATP stimulates AMP deaminase leading to uric acid and Pi release. The consequence of this is that there is a decrease of intracellular inorganic phosphate level and this limits the rate of ADP rephosphorylated to ATP, thus resulting in a decreased energy charge.
7. The decreased hepatic intracellular energy charge results in increased uric acid, lactate, and fructose release from the liver and subsequent lipid synthesis in the peripheral adipose tissues leading to obesity and hepatotoxicity.
8. In the fructose-loaded liver, the hepatocytes act as starving cells, sending signals (uric acid and Pi release) to the pancreatic β-cells and the hypothalamic–pituitary axis, resulting in increased catabolic hormone secretion (glucagon and corticosterone) (Szabó et al., 1995).
   a. Uric acid-treated mice exhibited significantly impaired glucose tolerance and lower insulin levels in response to a glucose challenge as well as an elevated level of uric acid which caused β-cell injury (Jia et al., 2013).
   b. Nakagawa et al. (2006) provided good experimental evidence for the causal role of uric acid in fructose-induced metabolic syndrome, by demonstrating that the inhibition of uric acid formation by allopurinol can prevent metabolic disorders caused by fructose feeding.
9. Relative to the starch diet, in this study the fructose diet increased the OTUs of both Firmicutes and Bacteroidetes, and decreased the proportions of Actinobacteria, Proteobacteria and Verrucomicrobia in the caecal microbiota.
10. Fructose, which is not absorbed, indirectly influences liver function by modifying the intestinal microbiota and thereby the microbial fermentation of fructose to VFAs providing substrates for lipid synthesis. Dietary nutrients seem to play a more significant role at the genus level than at the phylum level, which also likely changes the ratios of VFAs.
11. Fructose may also increase the absorption and the plasma level of LPS by altering the Gram-negative microbial population and thus contributing to inflammation and degradation of the mucosal barrier (Lambertz et al., 2017).

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